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DIAGNOSTIC AND PATHOPHYSIOLOGICAL ASPECTS OF CAPNOCYTOPHAGA CANIMORSUS INFECTIONS

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Diagnostic and pathophysiological aspects of Capnocytophaga canimorsus infections
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Dog and cat-related wound-infections in humans are common. *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* can be transmitted from dogs and cats to humans via bites or scratches. *C. canimorsus* is considered to be invasive and may cause sepsis, meningitis or endocarditis, whereas *C. cynodegmi* appears to be less virulent and is mainly associated with wound-infections. During 2007-2010, I collected clinical isolates of animal bite associated *Capnocytophaga* species, which was used in all projects included in this thesis. In study I, we used our collection of n=22 isolates to evaluate the performance of the recently introduced MALDI-TOF instrument and compared the results with the conventional typing method VITEK2. A species-specific PCR was established and used as the reference method. MALDI-TOF analysis identified all blood isolates as *C. canimorsus* and 13/14 wound isolates were identified as *C. cynodegmi*. Taken together, we found that MALDI-TOF was more accurate, faster and more cost-effective than conventional typing methods. In study II we analysed the interaction of human serum with *C. canimorsus* and *C. cynodegmi*. Whole blood and serum bactericidal assays unequivocally showed that both *C. canimorsus* and *C. cynodegmi* were sensitive to the bactericidal properties of whole blood and serum. In study III we continued to work with one of the strains in our collection, which could not be typed by the species specific PCR in study I. Whole-genome sequencing of the 22 *Capnocytophaga* species isolates in our collection revealed that the non-typable isolate in fact constituted a novel species, which we named "*Capnocytophaga stomatis*". Finally, in study IV we determined the antibiotic profiles against *Capnocytophaga spp*. Currently, there is a lack of comprehensive knowledge on the antibiotic susceptibility patterns for *Capnocytophaga spp.* Therefore, in study IV, we set out to study the antibiotic susceptibility profiles against *Capnocytophaga spp*. We found that two strains of *C. cynodegmi* and 2 strains of *C. stomatis* were resistant to amoxicillin-clavulanate, the first-line antibiotic therapy for animal bite related infections. Detailed analysis of whole genome data revealed that these strains carried a previously undescribed class D beta-lactamase gene with carbapenemase activity. Thus, our findings suggest that animal associated bacteria could serve as a potential reservoir for antibiotic resistance genes, which could have clinical implications when treating animal bite associated infections. Taken together, the data presented in this thesis may improve diagnosis, increase the understanding of the pathogenesis and facilitate correct antibiotic treatment of animal bite associated Capnocytophaga infections.

LIST OF SCIENTIFIC PAPERS

I. **Salah Zangenah**, Volkan Özenci, Stina Boräng and Peter Bergman. Identification of blood and wound isolates of *C. canimorsus* and *C. cynodegmi* using VITEK2 and MALDI-TOF. European Journal of Clinical Microbiology & Infectious Diseases, 2012,Volume 31, Issue 10, pp 2631–2637

II. **Salah Zangenah** and Peter Bergman Rapid killing of *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* by human whole blood and serum is mediated via the complement system. SpringerPlus, 20154:517

III. **Salah Zangenah**, Nasir Abbasi, Anders F. Andersson and Peter Bergman. Whole genome sequencing identifies a novel species of the genus *Capnocytophaga* isolated from dog and cat bite wounds in humans Scientific Reports, 2016, Article number: 22919 (2016)

IV. **Salah Zangenah**, Volkan Özenci, Anders F. Andersson and Peter Bergman. Genomic analysis reveals the presence of a class D beta-lactamase with broad substrate specificity in animal bite associated *Capnocytophaga* species. Submitted Manuscript

LIST OF ADDITIONAL PAPERS

- **Salah Zangenah**, Gülay Güleryüz, Stina Boräng, Måns Ullberg, Peter Bergman and Volkan Özenci. Identification of clinical *Pasteurella* isolates by MALDI-TOF —a comparison with VITEK 2 and conventional microbiological methods. Diagn Microbiol Infect Dis. 2013 Oct;77(2):96-8.

- **Salah Zangenah**, Linda Björkhem-Bergman, Anna-Carin Norlin, Susanne Hansen, Lars Lindqvist, Birgitta Henriques-Normark, Yenan Bryceson, Lars Nyman and Peter Bergman. The Pneumocell-study: Vaccination of antibody deficient patients with Prevenar13. Submitted Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AMPs	Antimicrobial peptides
CDC	Center for Disease Control and Prevention
DAI	DNA-dependent activator of IFN-regulatory factors
DAMPs	Damage-associated molecular pattern molecules
DF-2	Dysgonic fermenter type 2
DIC	Disseminated intravenous coagulopathy
ESBL	Extended-Spectrum β-Lactamase
MAC	Membrane attack complex
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
MBP	Mannan binding protein
MRSA	Methicillin-resistant Staphylococcus aureus
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization
PAMPs	Pathogen-associated molecular patterns
PRR	Pattern recognition receptors
RIG-I	Retinoid acid-inducible gene I
TLRs	Toll-like receptors

1 INTRODUCTION

Humans have been using animals for their meat, bones, skin, work, service, sports, companions, etc. To a certain extent, the interaction between animals and humans have been mutually beneficial , which generally have resulted in satisfaction for humans and sometimes unintentionally have caused suffering for the animals[1,2]. Naturally, the relationship between humans and animals has a prehistoric origin, a heritage from our ancestors. We learned that animals could be domesticated for different purposes, e.g. horses as workers and cats and dogs as companions[3,4].

Alongside these relationships, a microscopic interaction has been going on that certainly have affected humans. Pet animals are carriers of bacteria, viruses, parasites and fungi. These microorganisms can be transmitted to humans directly, e.g. via bites or scratches or indirectly, e.g. via a vector or via contaminated surfaces[5–8]. However, most microorganisms are harmless to humans, but some are zoonotic pathogens and potentially dangerous to humans, such as the notorious rabies virus, which cause many deaths across the world today[9]. The most common zoonoses are summarized in Table 1A and B[9–27].

Today, dogs and cats are the most common companion animals and together are responsible for the majority of pet-associated infections[28]. These animals carry many different microorganisms that do not belong to the human normal flora and our knowledge about most of them are very limited[29,8,30] Figure 1. In addition to the transfer of microorganisms from animals to humans, dog and cat-bites can cause significant physical damage, which can aggravate and spread the infection, sometimes resulting in fatal outcome[31–34].

Figure 1. A bacterial culture obtained from a dog's mouth. The growth pattern is typically polymicrobial and contains a large number of bacterial species.

Table 1A: Common bacterial and fungal zoonoses and their respective reservoir and vector (brackets).

Bacteria	**Fungi**
Actinobacillus lignieresii (horses)	*Arthroderma* species (cats)
Aeromonas hydrophila (fish)	*Basidiobolus ranarum* (horses, dogs, sheep)
Anaplasma phagocytophilum (tick)	*Blastomyces dermatitidis* (VAS)
Bacillus anthracis (grass-eating animals)	*Coccidioides* species (VAS)
Bartonella henselae (cats, fleas)	*Conidiobolus* species (horses, dogs, sheep)
Bergeyella zoohelcum (dogs, cats)	*Cryptococcus* species (VAS)
Bordetella pertussis (nonhuman primates)	*Emmonsia species* (rodents)
Borrelia burgdorferi (tick)	*Histoplasma capsulatum* (cattle, horses)
Brucella spp (VAS)	*Lacazia loboi* (dolphins)
Burkholderia spp (horse, mule)	*Mallassezia* species (VAS)
Campylobacter spp (mostly poultry)	*Microsporidia species* (VAS)
Capnocytophaga spp (dogs and cats)	*Microsporum* spp (VAS)
Chlamydophila psittaci (mostly birds)	*Paracoccidioides* spp (VAS)
Coxiella burnetii (VAS)	*Pneumocystis* spp (rodents, dogs, cats, cattle)
Ehrlichia chaffeensis (ticks)	*Sporothrix* spp (cats and VAS)
Erysipelothrix rhusiopathiae (VAS)	*Talaromyces marneffei* (bamboo rats, VAS)
ESBL[a] (livestock)	*Trichophyton* spp (VAS)
Francisella tularensis (rodents and VAS)	
Leptospira interrogans,spp (rodents,VAS)	
Leptospira spp (rodents)	
Listeria monocytogenes (VAS)	
Mycobacterium spp (VAS)	
Mycoplasma spp (livestock)	
Pasteurella spp (VAS)	
Plesiomonas shigelloides (fish)	
Rickettsia (tick,fleas)	
Salmonella spp (poultry, turtle, snake)	
Shigella spp (VAS)	
Spirillum minus (rats)	
Staphylococcus spp, MRSA[b] (VAS)	
Streptobacillus moniliformis (rat)	
Streptococcus equi (horses, mules)	
Streptococcus zooepidemicus (horse, mule)	

a = Extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* b = Methicillin-resistant *Staphylococcus aureus,* VAS = various animal species.

Table 1B: Common viral and parasitic zoonoses and their respective reservoir and vector (brackets).

Viruses and Unconventional agents	Parasites
Aichi virus (row oyster)	*Anisakis simplex* (fish)
Avian influenza (birds)	*Babesia microti* (tick)
Bunyavirus (mosquito)	*Clonorchis sinensis* (fish)
Chikungunya virus (mosquito)	*Diphyllobothrium latum* (fish)
Colorado tick fever virus (ticks)	*Echinococcus* spp (dogs and VAS)
Cowpox virus (cats and VAS)	*Fasciola hepatica* (fish,snail)
Crimean–Congo haemorrhagic fever virus (ticks and domestics animals)	*Fasciolopsis buski* (pigs, fish)
Dengue (mosquito)	*Giardia intestinalis* (VAS)
Dobrava–Belgrade virus (yellow-necked	*Heterophyes heterophyes* (fish)
Eastern equine encephalitis virus (mosquito)	*Leishmania donovani* (sand flies)
Ebola virus (mostly fruit bats and VAS)	*Opisthorchis viverrini* (fish)
Far eastern (ticks)	*Paragonimus westermani* (VAS)
Foot-and-mouth disease virus (VAS)	*Plasmodium falciparum/species* (mosquito)
Hendra virus (pteropid fruit bats)	*Pseudoterranova decipiens* (fish)
Hepatit E-virus (pigs)	*Schistosoma japonicum/species* (infected snails in water)
Herpesvirus (macaques)	*Taenia solium* (pigs)
Japanese encephalitis virus (mosquito)	*Toxocara canis*, *T. cati* (cats, dogs)
Lassa virus (rodents)	*Toxoplasma gondii* (cats)
Lyssaviruses, rabies virus (mostly dogs, other infected animal)	*Trichinella* spp (pigs)
Monkeypox virus (macaque)	
Nipah virus (pigs, bats)	
Paramyxovirus type 1 (birds)	
Parapoxvirus (sheep and castles)	
Powassan virus (ticks)	
Prion (Cow,Creutzfeldt-Jakob Disease)	
Puumalavirus (rodents)	
Rift Valley fever (domesticated animals)	
Ross River virus (mosquito)	
Rotavirus (pigs)	
Sochi virus (Black Sea field mouse)	
Tick-borne encephalitis virus	
Venezuelan equine encephalitis virus (mosquito)	
Vesicular stomatitis virus (mosquito)	
West Nile virus (mosquito)	
Western equine encephalitis virus (mosquito)	
Zika virus (mosquitoes)	

VAS = various animal species.

1.1 How common are animal bite infections?

Many of us have or will experience a bite from a pet animal. In USA alone, there are approximately 75 million dogs and 57 million cats. Five million incidences of animal bite injuries are reported per year and ca 1% of them require medical care[15,35].

In Sweden, there are 784 000 dogs and 1 159 000 cats (Svenska Kennelklubben, Statistiska centralbyrån 2012). According to the Swedish National Board of Health and Welfare (Socialstyrelsen) the absolute majority of all reported animal bites are caused by dogs (80-90%) and a smaller portion are caused by cats (3-15 %). A cat-bite is usually deeper and thus more often associated with an infection. For cat-bites, 30-50% of all cases lead to an infection, whereas 2-25% of all dog bites are estimated to cause an infection[15,35,36].

1.2 Animal bites – risk factors and medical seeking behaviour

There are several factors that affect the outcomes after dog- and cat-bites. The size of the animal has great impact on the type and the severity of the physical injury. Generally, a big dog causes larger tearing and crushing injuries than smaller dogs. Cats more often cause puncture injuries, with a high risk of subsequent infection[33].

A prospective study over 5000 cases showed that male and female individuals are at equal risk for animal and human bites. Bites of dogs (48%), cats (18%), humans (8%), other animals (7%) and human self-bites (19%) were responsible for these injuries and risk for infections after cat bites were estimated to be six times higher than after a dog-bite[37].

Most patients with superficial wounds do not seek medical attention, whereas those with severe injuries need acute medical attention and sometimes are even transported with an ambulance to hospital. A third category comprises the "over-anxious" patient who seeks medical care immediately after the incident. Patients with superficial wounds more often seek medical care when the signs of inflammation and infections have developed. Combined, the medical seeking behaviour of animal-bitten individuals introduces a bias in the reporting systems and generally it is believed that the number of mild injuries after contact with cats and dogs is underestimated[7].

1.3 Wound cultures – a microbiologist's view

In addition to the physical injuries, infections can occur after animal bites. These infections can range from superficial and localized wound infections to severe life threatening systemic infections, such as sepsis[38–40]. The microbial etiology is important since it will guide the antibiotic treatment. To obtain a wound specimen for bacterial culture is a well-established standard procedure in most healthcare systems worldwide[41]. Clinical bacteriological laboratories use standard media, such as agar plates and enrichment broth culture to support growth of wound associated bacteria. Most of these wounds are colonized by aerobic and anaerobic bacteria Figure 2. The most common aerobic bacteria associated with dog and cat bites are *Pasteurella* species, *Staphylococcus* species, *Streptococcus* species, *Neisseria* species, *Corynebacterium* species, *Moraxella* species, *Enterococcus* species, *Bacillus* species and *Pseudomonas* species. The most common anaerobic bacteria are *Fusobacterium species*, *Bacteroides* species. *Porphyromonas* species, *Prevotella* species, *Propionibacterium* species and *Peptostreptococcus* species[42,43].

Figure 2. Bacterial culture of a wound specimen after a dog bite wound, showing polymicrobial growth, including a mixture of human commensal bacteria and dog oral flora.

The focus of this thesis is *Capnocytophaga species*, e.g. *Capnocytophaga canimorsus* and *C. cynodegmi*, which may be transmitted from dogs and cats to humans. *C. canimorsus* is mainly associated with systemic infections, whereas *C. cynodegmi* is mostly associated with wound infections, although a few cases of *C. cynodegmi* peritonitis have been reported[44–50]. In 1961 the Center for Disease Control and Prevention (CDC) received the first strain of *C. canimorsus* that was isolated from the blood of a splenectomised boy. From 1961 to 1975 CDC received in total 17 blood culture isolates (CDC collection 1961- 1975) with similar. phenotypic patterns. These bacteria were classified as "dysgonic fermenter type 2 (DF-2)"[48,51,52]. In 1976, Bobo and Newton published the first case report "A previously

undescribed gram-negative bacillus causes septicaemia and meningitis"[53]. It was first in 1989 that Don J. Brenner genetically and phenotypically characterized DF-2 and"DF-2 like" bacteria and the names *Capnocytophaga canimorsus* and *C. cynodegmi* were proposed for DF-2 and "DF-2 like", respectively[51] .*Capnocytophaga* is derived from Latin; bacteria requiring (eating) carbon dioxide. *Canimorsus*: Cani; dog, morsus; bite and *Cynodegmi* is derived from Greek: Kyno; dog, degmos; bite[51]. In addition to *C. canimorsus* and *C. cynodegmi*, *Capnocytophaga canis* has recently been described as a member of the normal flora of the oral cavity of healthy dogs[54].

The prevalence of *C. canimorsus* and *C. cynodegmi* in dogs and cats has been determined. *C. canimorsus* can be found in 74% of dogs and 57% of cats and *C. cynodegmi* can be found in 86%-96% of dogs and in 84% of cats[55,56].

1.4 Epidemiology and clinical records

The epidemiological pattern of the first sepsis cases (CDC collection 1961- 1975) were as follows: out of 17 patients, 88% were males, 65% were over 40 years, 30% were splenectomised, and 24% had a history of alcohol abuse. In addition, 59% had a history of a dog bite and 24% had a history of a reported contact with a dog or other animals[57]. After the initial characterization of *C. canimorsus* by Brenner (1989)[51], several follow-up reports have confirmed that *C. canimorsus* indeed can cause severe invasive infections. Most reports show that *C. canimorsus* can be transmitted via bites, scratches or via wounds caused by sharp objects, such as the teeth of the animal. The incubation period before clinical symptoms are present can range from hours to more than one week. Symptoms are similar to those occurring in sepsis, such as fever, chills, lethargy, myalgia, diarrhoea, and nausea and vomiting. Notably, signs of local wound infections are often not observed[48,51,58].

A summary of 2 review papers comprising a total of 99 clinical cases of *C. canimorsus* sepsis showed that 57% of the cases had a history of a dog bite, 3% had a history of a cat bite and 10-27% were exposed for a dog without being bitten. Interestingly, 11%-17% did not admit any animal contact and one individual had been exposed for another animal than a dog or a cat. Approximately 1/3 of the sepsis patients were previously healthy individuals, 10-33% were asplenic patients, 18-22% had a history of alcohol abuse and 5% were treated with corticosteroids. Sepsis was more common in males (70-74%) than in females (25-30%) [59,60]. The most common clinical manifestations of *C. canimorsus* sepsis included fulminant septicaemias with fatal outcome, disseminated intravenous coagulopathy (DIC), meningitis, endocarditis and endophtalmitis[33,42,47,48,61–63]. Naturally it would be expected that most *C.*

canimorsus infections should be related to wounds. Unexpectedly, this organism is in fact rarely isolated from infected wounds, but rather from blood cultures[48]. Another interesting aspect is that despite the fact that children are frequently victims of cat and dog bites, especially children aged 5-9[7], there are no case reports on infections caused by animal *Capnocytophaga* in children. However, there are 2 case reports of *Capnocytophaga* sepsis in infants, a 12-day-old baby and in a 3-month-old new-born baby[48,59,60].

1.5 Bacteriology

The phenotypic profile of DF-2 as originally stated by Brenner *et al,* showed that DF-2 was a slow growing, gram-negative bacilli that required a long incubation time (more than 24h) in CO_2 atmosphere. It was unable to grow on MacConkey agar. DF-2 was positive for oxidase and catalase, glucose, maltose, and lactose and gliding motility test and negative for sucrose and mannitol, indole, urease, and nitrate. Most of these tests are obsolete today but especially the oxidase and catalase tests have been proven to be very useful also today. *C. canimorsus* and *C. cynodegmi* are positive for oxidase and catalase, whereas human *Capnocytophaga* species are negative for these tests. In 1989 Don J. Brenner genetically and phenotypically characterized DF-2 and DF-2-like bacteria and proposed the names *Capnocytophaga canimorsus* and *C. cynodegmi,* respectively. *C. cynodegmi* differs from *C. canimorsus* by having the ability to ferment sucrose, raffinose, inulin, and mellobiose[48,51] Table 2.

Table 2: Biochemical characteristics of *Capnocytophaga* species.

Property and Characteristic	***C. canimorsus***	***C. cynodegmi***	***Capnocytophaga* species**
Habitat	animal	animal	human
Growth Requirements	capnophilic (5% CO_2) mesophilic (30-37^oC)	capnophilic (5% CO_2) mesophilic (30-37^oC)	capnophilic (5% CO_2) mesophilic (30-37^oC)
Gram stain	negative	negative	negative
Cell shape	fusiform rod	fusiform rod	fusiform rod
Sporulation	none	none	none
Gliding motility	gliding	gliding	gliding
Hemolysis, SBA	negative	negative	negative
Growth on MacConkey agar	negative	negative	negative
Indole	negative	negative	negative
Urease	negative	negative	negative
Lysine decarboxylase	negative	negative	negative
Ornithine decarboxylase	negative	negative	negative
NO_3 to NO_2	negative	variable	variable
NO_2 to N_2	variable	variable	negative
Hydrolysis of esculin	variable	positive	variable
Hydrolysis of ONPG	positive	positive	variable
Arginine dihyrolase	positive	positive	negative
Oxidase	positive	positive	negative
Catalas	positive	positive	negative
Acid production from			
Inulin	negative	positive	negative
Sucrose	negative	positive	positive
Raffinose	negative	positive	variable
Melibiose	negative	positive	negative
Glucose	positive	positive	positive
Maltose	positive	positive	positive
Fructose	variable	positive	variable
Lactose	positive	positive	variable
Xylose	negative	negative	negative
Mannitol	negative	negative	negative
Mannose	variable	positive	positive
Galactose	positive	variable	variable
Hydrolysis of glycogen	positive	variable	variable
Hydrolysis of Starch	positive	positive	variable

1.6 The immune system and *Capnocytophaga* species

The immune system is classically divided into innate and adaptive immunity. These systems work together to detect foreign substances, eliminate pathogens, control the microbiota and are involved in maintaining homeostasis within the body. The immune system cleverly fights and controls the microorganisms to protect the host. Microorganisms in turn have successively evolved a number of different strategies to avoid the immune system, to be part of the microbiota or to become a potential pathogen[64–67]. Our contact with bacteria begins early; it´s believed that the first contact takes place during birth, likely also during the intrauterine life[68–70]. To simplify matters, the contact between the microbe and the host can be of two types, inflammatory and non-inflammatory. The inflammatory component occurs when a bacteria is crossing host barriers and triggers an immune response. In contrast, the non-inflammatory interaction is best exemplified by the normal flora and its interaction with the human immune system. Here, a balance is maintained and this commensalism is beneficial for both the host and the microorganism; e.g. the host is protected by harmless bacteria and the bacteria gain nutrients for growth. Notably, disease may occur when this delicate balance is disturbed, such as during injury, exposure to cytostatic drugs or after treatment with broad spectrum antibiotics[65,71,72].

Infection can also occur when presumably harmless bacteria end up in the wrong location. This is one way to look upon infections with *Capnocytophaga* species, which normally are found in the oral flora of healthy dogs and cats. When these bacteria are transferred to humans via bites or scratches, a clinical infection is often the result. There is no evidence that *Capnocytophaga* species has the potential to cause invasive infections if the skin or mucus barrier is intact. Intact skin and mucous membranes are considered as the first line of defences, constituting physical and biochemical defences against invading microorganisms. These parts of the immune system constitute a rapid response to invaders and in general resulting in the elimination of the invaders. In addition to serving as physical barriers, the skin and mucosal surfaces of humans are colonized by large microbial communities, which interact with immune system and contribute to the control of invaders[38,73–77].

The skin is a very complex organ that contains several cell-types, such as keratinocytes, fibroblasts and dendritic cells. Upon recognition of a microbe, these cells produce cytokines and antimicrobial peptides (AMPs), which is the first response to a microbial assault. For example, a fissure caused by sharp object e.g. animal teeth, can trigger an acute innate immune response. Subsequently damage-associated molecular pattern molecules (DAMPs) from the host and pathogen-associated molecular patterns (PAMPs) trigger pro-inflammatory

reactions. Through pattern recognition receptors (PRRs), like Toll-like receptors on keratinocytes, PAMPs and DAMPs will be recognized, which results in production of AMPs, the release of chemokines and cytokines, followed by subsequent recruitment and activation of macrophages, neutrophils and other leukocytes. These cells and proteins work together to eliminate bacteria, remove dead tissue and debris, and finally aid in the wound-healing process[74,75,78].

1.7 Recognition by innate sensors

Until now several of PRR have been discovered such NOD-like receptors (NLRs) and Toll-like receptors (TLRs) which are expressed on e.g. macrophages and dendritic cells. TLRs are transmembrane receptors that can recognize fragments of microbes and other antigens such as cell debris after injuries. Peptides, nucleic acids and lipid-based molecules have been described as ligands for TLRs. In humans, ten TLRs, TLR1-TLR10, have been discovered. Different types of TLRs sense different PAMP fragments; TLR1, TLR2, TLR4 and TLR6 recognize lipid-related structures, whereas TLR3 and TLR7-9 recognize nucleic acids. Moreover retinoid acid-inducible gene I (RIG-I), DNA-dependent activator of IFN-regulatory factors (DAI) and two nucleotide-binding oligomerization domains (NOD)-like receptors (NOD1 and NOD2) are intracellular PRRs that can trigger the immune response to intracellular PAMPs and DAMPs[78–81].

1.8 The complement system

It is well known that human serum has a good bactericidal capacity, in particular against gram-negative bacteria[82–85]. This bactericidal activity involve antibodies (heat stable component of serum) and the complement (heat labile component of serum)[86–89].

The complement system consists of more than 32 components, functioning in the innate and adaptive immune systems with a wide range of functions, including antimicrobial activity, chemotaxis and activators of immune cells to control inflammatory reactions. Thus, the complement system works to “complement” the human immune system. In addition, the complement-system is involved also in other processes, such as angiogenesis and synapse maturation[90,91].

The activation of the complement system is divided into three pathways: the classical, the alternative and the lectin pathway. These three pathways activate the lytic pathway, also called the membrane attack complex (MAC)[85,92,93].

1.8.1 The classical pathway

The classical pathway is generally activated by binding of C1, that consist of subunit proteins C1q, C1r and C1s, to the FC region of IgM or IgG clusters that have bound antigen; this activation is known as the antibody-dependent pathway[82,88,94]. The subunit C1q is a pattern recognition molecule (PRM) and can activate the classical pathway by direct binding to structures of microorganisms and apoptotic cells. C1q can also activate the classical pathway by binding to other PRMs, such as C-reactive protein[95]. The binding of C1q activates C1r and C1s, which results in formation of C1qrs, a formation that is highly dependent on the presence of calcium-ions to form[96]. Subsequently, C1qrs cleaves the substrates C4 and C4b bound C2 to form C4b2a (C3 convertase)[92,97–100].

1.8.2 The alternative pathway

This pathway is activated by binding of the two factors B and D to hydrolysed C3 (C3i), which results in formation of C3 convertase (C3bBb). The formation of C3bBb generates more C3b, which amplifies the response. Subsequently C3b covalently binds to the surface of pathogens, which results in opsonisation of the pathogen and formation of MAC[92,101,102].

1.8.3 The lectin pathway

The discovery of mannan binding protein (MBP), a calcium-dependent lectin, in 1978 resulted in the discovery of the lectin pathway[103]. It involves the sugar binding molecules (lectins) Mannose-binding lectin (MBL) and ficolins 1-3, which function as PRMs. These molecules bind to carbohydrates (mannans) on surfaces of pathogens. Similar to C1q these PRMs associate with two MBL-associated serine proteases, MASP-1 and MASP2 after binding to the antigen. Formation of MBL/MASP-1/MASP-2 complex results in the cleaving of C4 and C2 by MASP-2, which subsequently results in formation of a C3 convertase. C3 convertase cleaves C3 into C3b and C3a. Finally, the binding of C3b to C4bC2a results in formation of C4bC2aC3b, a C5 convertase[99,104–106].

1.8.4 The membrane attack complex

One of the most important consequences of complement activation is the formation of C5 convertase, an enzyme that mediates the cleavage of C5 and the formation of the membrane attack complex (MAC) on the surface of pathogens. Formation of MAC on the surface of pathogens, e.g. gram-negative bacteria, results in pores in the lipid bilayer, which cause a loss of membrane potential and lysis of the pathogen[85].

1.9 The macrophage response to bacteria

Macrophages are one of the most important effector cells of the innate immune system and provide crucial protection against pathogens. Embryo-derived resident macrophages and monocytes recruited to the site of infection can act as pro-inflammatory macrophages, active phagocytic cells (M1) or they can undergo changes to act as anti-inflammatory macrophages (M2), which are implicated in tissue repair. In other words, neither monocytes nor macrophages are a homogeneous population of cells. Macrophages have a wide range of functions, in addition to their phagocytic capacity. For example, they produce cytokines and function as antigen-presenting cells (APC), which makes macrophages an important link to the adaptive immunity. Resident and inflammatory macrophages exist in many different organs and depending on localization and function these macrophages also have specific names, such as alveolar macrophages and liver Kupffer cells [74,107,108]. Phagocytic capacity is the most eminent property of macrophages Figure 3. After engulfing bacteria into their phagosomes, these phagosomes fuse with lysosomes and other vesicles, which contain antimicrobial effectors, such as AMPs and proteases, to mature into phagolysosomes, a very harsh environment for most bacteria. But some pathogens, such as *Mycobacterium tuberculosis* have evolved strategies to avoid phagolysosomes, and can move into the cytoplasm before the formation of phagolysosomes occurs. Finally, after having fulfilled their task, macrophages are programmed to undergo apoptosis in order to maintain homeostasis within the body[74,109,110].

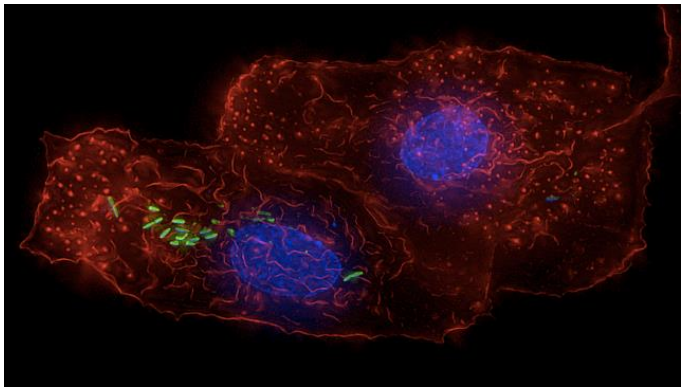

Figure 3. *C. canimorsus* (green = CFSE) inside of macrophage (red = actin is stained with Phalloidin and blue = nucleus is stained with DAPI), possibly exploiting the intracellular niche as a hiding place from complement activity. (Zangenah *et al*, unpublished).

1.10 The cellular immune response to *Capnocytophaga canimorsus*

Generally, the interaction between *C. canimorsus* and immune-cells are poorly described. In 1995 Fischer *et al* published a study on the interactions between *C. canimorsus* and *C. cynodegmi* with J774 mouse macrophage cells[111]. By electron microscopy, they found *C. canimorsus* and *C. cynodegmi* within the vacuoles of the macrophages after 48h and 72h, respectively. Further, they claimed that the bacteria replicated intracellularly, since there was evidence of more than one bacterial cell within the vacuoles and bacterial septation was observed on electron micrographs. In addition, a cytotoxic effect was observed for *C. canimorsus*, whereas this property was not observed for *C cynodegmi*. The cytotoxic effect was not induced by endotoxin, according to this study[111].

The next study on *C. canimorsus* and the interaction with immune-cells was not published until 2007, when Shin *et al* published data on the inflammatory response of human and mouse macrophages in response to *C. canimorsus* infection[112]. They found that *C. canimorsus* could survive and multiply in the presence of J774.1 mouse macrophage cells, and they could not observe any bacterial internalization. Further, no evidence of cytotoxicity was found in this study, which was in contrast to Fisher *et al*[111,112] .Interestingly, infected J774.1 mouse macrophages did not release tumour necrosis factor (TNF)– α or IL-1 α. Cells infected with either live or heat-killed *C. canimorsus* (Cc5), isolated from a patient with fatal septicaemia, produced low levels of IL-6 and IL-10 and *C. canimorsus*-infected THP-1(human monocytic cell line) did not release detectable pro-inflammatory cytokines, IL-1β, IL-6, IL-8, IFN-γ, TNF- α and macrophage inflammatory protein–1β, suggesting that *C. canimorsus* somehow could avoid immune recognition. Based on these results, Shin *et al* proposed that *C. canimorsus* exhibited several potent immune-evasive properties, which combined could contribute to the severe presentation often observed in *C. canimorsus*-sepsis[112].

In 2008, a follow-up paper from the same group found that mouse macrophages were highly ineffective in taking up and killing *C. canimorsus*. Pre-opsonisation with complement did not increase the killing effect whereas pre-opsonisation with rabbit sera specifically raised against *C. canimorsus* enhanced the killing. Interestingly J774.1 macrophages treated with conditioned medium of *C. canimorsus* bacterial cultures did not kill *E. coli*, suggesting that there was an anti-phagocytic factor present in *C. canimorsus* supernatants. Shin *et al* showed that *C. canimorsus* (strain Cc5) could block the pro-inflammatory response. They found that only 2 of 10 *C. canimorsus* strains could inhibit the pro-inflammatory response (TNF and NO release) in J774.1 cells after a challenge with heat killed *Y. enterocolitica* [113].

Shin *et al* reported in 2009 a study on the interaction between *C. canimorsus* with the human complement system and human polymorphonuclear leukocytes (PMNs). They showed that three *C. canimorsus* strains were highly resistant to 10% normal human serum and one strain was resistant also to serum from dog and mouse. It was concluded that the complement resistant property was a common property of *C. canimorsus*. Additionally Shin *et al* reported that human PMNs could not take up or kill *C. canimorsus* (one strain, Cc5, was tested). Interestingly, a mutant of Cc5, with a gene-deletion of the enzyme glycosyltransferase, was sensitive to PMN uptake and killing as well as susceptible to complement mediated killing via the antibody-dependent classical pathway[114].

1.11 *Capnocytophaga species* and human serum

There are only a few studies that have described the interactions between animal *Capnocytophaga* species and human serum. The focus in previous studies has been on *C. canimorsus* and up to this point; it is not clear whether *C. canimorsus* is resistant or sensitive to human serum. The first report from Butler *et al* showed that *C. canimorsus* was resistant to rabbit serum[115] whereas Hicklin in 1987 showed that *C. canimorsus* was susceptible to normal human serum[116]. In contrast to Hicklin, Shin *et al.* in 2009 showed that *C. canimorsus* was resistant to normal human serum[114]. Clearly, it is not easy to draw firm conclusions from these studies. This lack of knowledge on the interaction between *C. canimorsus* and *C. cynodegmi* with human serum was the rationale for my own work presented in study II where I found that all tested strains were indeed killed by human serum in a complement dependent way.

1.12 Clinical examples of a deficient complement system

Regardless of the susceptibility of *Capnocytophaga species* to bactericidal activity of normal serum, there are conditions that are associated with low levels of complement factors or that impair complement system activity. For example, patients with congenital or acquired splenia or with a dysfunctional spleen constitute 1/3 of the cases with *C. canimorsus* sepsis. Asplenic patients have an increased risk of post-splenectomy infections and these patients have low serum levels of IgM, which is a highly important activator of the classical pathway. Notably, it has been shown that IgM memory B cells decline after splenectomy[117]. The most common complement deficiencies are: Classical Pathway Deficiencies: C1q, C1s, C2 and C4 deficiency. Lectin-Pathway Deficiency: MBL deficiencies, L-ficolin. Alternative Pathway Deficiency: Factor B, factor D and Properdin deficiency. These conditions have in common that they predispose the patient to various bacterial infections. The most striking example is

probably C2-deficiency, which is strongly connected to an increased risk for meningococcal infection[87,100,118–120]. However, an early diagnosis can initiate full vaccination against meningococci and antibiotic prophylaxis, which will be of great clinical value for the individual patient. Importantly, there has been little research into the role of these complement disorders and their connection to invasive *Capnocytophaga* species infections. Our finding in paper II, where we clearly show that the complement system is a major factor in killing *Capnocytophaga* species in human serum, could form a platform for future studies in this area.

2 AIMS

The general aim of the thesis was to study a collection of animal bite associated *Capnocytophaga* species with regard to diagnostic methods, interactions with the immune system, genomic relationships and antimicrobial susceptibility patterns.

Study I

The aim of study I was to evaluate MALDI-TOF as a novel diagnostic method in the identification of animal bite associated *Capnocytophaga* species.

Study II

The aim of study II was to investigate the interaction between human whole blood and serum with *C. canimorsus* and *C. cynodegmi* and specifically to test whether these bacteria could evade the complement system.

Study III

The aim of study III was to describe the genomic relationships within the *Capnocytophaga* family by whole genome sequencing and phylogenetic analyses.

Study IV

The aim of study IV was to provide a comprehensive and detailed description of the antimicrobial susceptibility patterns of animal bite associated *Capnocytophaga* species.

3 METHODS

In total we had a collection of 22 clinical isolates of *Capnocytophaga* species collected during the years 2005-2010. The reference strains *C. canimorsus* (ATCC 35978, blood isolate), *C. cynodegmi* (ATCC 49045, wound isolate) were used as controls in all studies. For experiments related to the complement system two *Salmonella* strains; SL3769 with (rfaG-gene mutant, serum sensitive) and SL3770 (parent strain, serum resistant) were used as controls (paper II).

3.1 Isolation of *Capnocytophaga* species from wound specimens

Wound swab samples were taken from specimens where there was a history of a dog or cat bite or physical contact with any of these animals. According to routine laboratory procedures, these specimens (swabs) were inoculated on four agar plates (haematin, blood x 2 and CLED). The plates were incubated in CO_2, aerobic and anaerobic atmosphere for 48 h. The blood- and haematin-agar plates of randomly selected cultures had extended incubation times, up to 5 days in 35-36^{o}C, 5% CO_2, which is normally required for Capnocytophaga to form typical colonies (Figure 4A). If a colony was suspected to be a Capnocytophaga, each colony was subjected to Gram staining and further sub-cultured. After 48-72 h of incubation in 35-36^{o}C and 5% CO_2, visible colonies were formed. Selected colonies were tested for catalase and oxidase. The criteria for *Capnocytophaga* species were as follows: typical cell morphology in microscopy (Figure 4B), i.e. fusiform, gram negative bacilli with pointed ends, positive in catalase and oxidase tests. Those isolates that fulfilled these criteria were stored in glycerol stocks at −70°C. Prior to subsequent experiments a small amount of the frozen isolate was sub-cultured on haematin or/and blood agar and incubated at 35-36^{o}C, 5% CO_2.

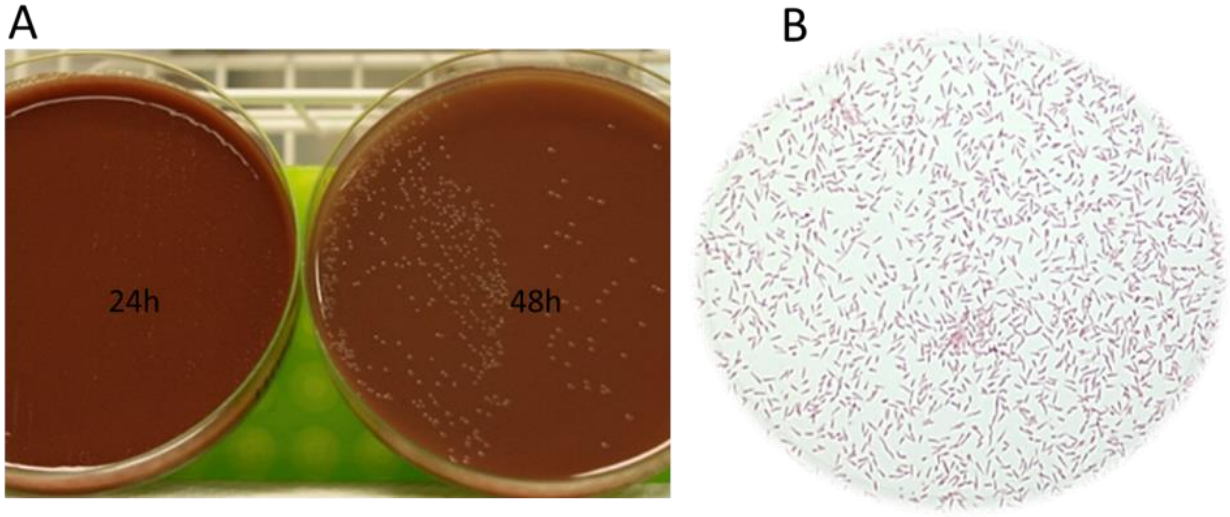

Figure 4A and B. Colonies of *C. canimorsus* are visible after 48 h incubation on haematin agar plates (A). Gram staining of *C. canimorsus* shows gram negative, fusiform and rod-shaped bacteria (B).

3.2 Collection of *C. canimorsus* from blood culture specimens

All *C. canimorsus* strains were recovered from blood culture systems at Falun Central Hospital and Karolinska University Hospital. According to standard procedures at the respective laboratory in Falun Central Hospital and Karolinska University Hospital, Gram staining and bacterial cultures were carried out for all positive blood cultures on blood- and haematin-agar plates and incubated in CO_2, aerobic and anaerobic atmosphere for a minimum of 48 h.

3.3 Identification of *Capnocytophaga* species by traditional methods

As described above, the collection used in this thesis was mainly identified based on colony morphology and two biochemical tests (catalase and oxidase). These methods are inexpensive and not too sophisticated, but have helped microbiologists to describe bacterial species for many years. In general, this diagnostic paradigm works well for fast growing bacteria (*Staphylococcus* species, *Streptococcus* species and *Pseudomonas* species, for example) and can identify the most common and clinically significant bacteria to the species level. However, a more detailed identification of fastidious bacteria, such as Capnocytophaga, requires additional methods. Traditionally, an extended panel of biochemical tests has been used. This was also true for the original identification of *Capnocytophaga canimorsus* [51].

Based on gram staining, oxidase and catalase tests, I could distinguish animal bite associated *Capnocytophaga* strains from human *Capnocytophaga* species, which are oxidase and catalase negative. To distinguish *C. cynodegmi* from *C. canimorsus*, sucrose, raffinose, and

mellobiose were used. These tests were performed according to the reference-method and a heavy inoculum was used for the fermentations tests, the incubation time was extended up to 5 days and the incubation occurred in CO_2 atmosphere, as recommended in the original description[51]. Despite careful and detailed analysis, we found most of these tests to be very slow, unreliable and not particularly useful in a clinical context.

The problems that we confronted with conventional methods were the main rationale for study I, where we wanted to improve the diagnostic procedures and evaluated the performance of MALDI-TOF in the identification of *Capnocytophaga* species.

3.4 MALDI-TOF mass spectrometry analysis

MALDI-TOF mass spectrometry (MS) has been used for decades in the analysis and characterization of biomolecules, such as lipids, DNA, proteins and peptides. In recent years MALDI-TOF has been used extensively in clinical laboratories with a wide range of applications, such as the identification of bacteria and other microorganisms[121]. In study I we evaluated Microflex LT MALDI-TOF system (Bruker Daltonics) and compared its performance with traditional methods that have been used in clinical microbiology laboratories for identification of *Capnocytophaga*. A small amount of bacterial colonies were collected with plastic loops and applied to a MSP96 polished steel target plate. Next, the samples were covered with 2.0 µl of the matrix (saturated α-cyano 4-hydroxy cinnamic acid in 50 % acetonitrile with 2.5 % tri flouro acetic acid) and analysed immediately after dryness of the sample in MSP96. To collect mass spectra, the FlexControl 3.0 software (Bruker Daltonics) with the manufacturer's pre-settings was used (spectra of m/z range of 2,000 to 20,000 Daltons). Next, the software MALDI Biotyper (Bruker Daltonics) was used to automatically compare the obtained spectrum with a reference spectrum from the database provided by the manufacturer. The spectra of the unknown samples were compared with the reference spectra and interpreted by the software. A reliable identification was presented as a score; a score greater than 1.9 was considered as a correct identification of bacteria to the species level and a score of 1.7 to 1.999 considered as a correct identification to the genus level. Importantly, the main limitation of the system is the database. If a bacterial species is rare and the database contains few reference spectra, there is a lower chance to obtain a high score. This was the fact for us when working with paper I. The database only contained one spectrum for *C. canimorsus* and one spectrum for *C. cynodegmi*, which resulted in low scores for most isolates.

3.5 Whole blood killing assay

The classical whole blood bactericidal assay, like serum killing assay, is based on the colony counting method, which measures the growth of bacteria in the presence of normal human whole blood. In these assays we use heparinized blood from healthy volunteers. Notably, some of the most common anticoagulants, such as citrate and EDTA, should not be used since these agents can bind calcium in the blood and serum, which would result in the inactivation of calcium dependent activities (complement activation) of whole blood and serum.

For the experiments involving whole blood, 900 µl of whole blood (heparinized) was mixed with 100 µl bacteria diluted in PBS. These tubes were incubated at 37^{o}C, using an end-over-end rotator at 6 rpm. After 90 min of incubation, the bacteria-blood solution was plated and after over-night incubation, viable bacteria were counted.

3.6 Serum killing assay

Normal human sera (NHS) from healthy individuals were pooled and used as serum source for most of the experiments. Full complement analysis on pooled NHS was performed by the Karolinska University Laboratory (Clinical immunology) to confirm the quality of the sera.

Several controls were used in these experiments (paper II): a) Heat inactivated serum. Complement-mediated killing of bacteria is heat sensitive which can be inactivated by heating serum for ca 30 min at 56^{o}C); b) C1q depleted serum was obtained from Sigma (classical pathway is inactivated); c) Serum from MBL deficient patients were obtained from the Immunodeficiency Unit, Karolinska University Hospital, Huddinge in relation to another study (ethical approval number 2011/116-31/4). d) EGTA-treated serum. Treating NHS with 10 mM EGTA-MgCl inhibits the calcium-dependent classical and lectin pathway[122–124].

The classical serum killing assay is based on the colony counting method, which measures the growth of bacteria in the presence of normal human serum. We used NHS diluted from 5% to 90% in phosphate buffer solution (PBS). Depending on the serum volume either Eppendorf tubes or 96 well microplates were used for the experiments. The growth of *Capnocytophaga* in most nutrient liquid broth, such as brain heart infusion (BHI)-broth was insufficient and varied in yield. Therefore we directly harvested *Capnocytophaga* from agar plates and dissolved them in PBS, which is a common procedure for fastidious bacteria[88]. A strain was defined as "serum-sensitive" if the viable counts decreased with more than 1_{10}log compared with the corresponding control.

3.7 DNA–DNA hybridizations

Thermo Scientific GeneJET Genomic DNA Purification Kit was used to isolate DNA from 24 strains of *Capnocytophaga* strains, (our collection including two *Capnocytophaga* references). Next, the DNA of these strains was subjected to whole genome sequencing at Science for Life Laboratory, Solna, Sweden. Based on genome sequencing, we studied *in silico* the relatedness between these strains by using DNA–DNA hybridization (DDH) method. Within microbiology DDH has is useful when 16S rRNA gene sequence similarities are too high to distinguish closely related organisms correctly. We used the web server to compare the genomes of all strains. The genome of each bacterial isolate was analysed against genomes of the other bacteria in the collection. DDH data was obtained by using the web server Genome-to-Genome Distance Calculator[125].

3.8 Antibiotic susceptibility testing

The Etest (BioMerieux) was used to determine the susceptibility of *Capnocytophaga* species to several clinically relevant antibiotics. The Etest is the gold standard to determine Minimum Inhibitory Concentration (MIC), in particular for resistance testing of slow-growing and fastidious bacteria. Prior to the resistance testing, the isolates were sub-cultured from frozen stocks onto blood agar for 48 hours, 35^{o}C in CO_2. Next, the isolates were harvested and suspended in sterile PBS to a McFarland standard of 0.5. The suspension was spread on blood agar and after dryness (when the suspension had been absorbed into the agar) a single Etest strip was applied on agar plate. The agar plates were incubated for 48 hours at 35^{o}C in CO_2. The MIC values were obtained by recording the zones of inhibition. Finally, the MIC values were interpreted according to the EUCAST antimicrobial guidelines for non-species related breakpoints (http://www.eucast.org/clinical_breakpoints).

3.9 Identification of antibiotic resistance genes

To identify antimicrobial resistance genes the web based program ResFinder from the Center for Genomic Epidemiology was used. The threshold for ID was set to 98% and the minimum length was set to 60%[126].

4 RESULTS AND DISCUSSION

4.1 Study I

Rationale: The conventional way to diagnose *C. canimorsus* and *C. cynodegmi* was slow, unreliable and not useful in a clinical bacteriological laboratory.

Aim: To evaluate MALDI-TOF as a novel way to identify *C. canimorsus* and *C. cynodegmi*.

Main results: We could show that the classical methods gram staining, oxidase and catalase could be used to identify animal Capnocytophaga to preliminary ID, but not to the species level. Notably, methods based on biochemical tests or fermentation tubes, including the automated typing system VITEK2, could identify some isolates to the genus level but were unreliable and ineffective in the identification of Capnocytophaga to species level.

We also found that *C. canimorsus* and *C. cynodegmi* could not be distinguished by 16S rRNA sequencing and all strains were identified as *C. canimorsus*/*cynodegmi*. This problem prompted us to establish a species specific PCR, which enabled us to identify these strains to the species level with a high accuracy[127].

Importantly, MALDI-TOF MS analysis identified blood isolates as *C. canimorsus*, whereas most wound isolates were identified as *C. cynodegmi*.

Notably one strain (W13) was not identified to the species level by using these methods, which was further explored in paper III (below).

Discussion: *Capnocytophaga* species are common bacteria present in the oral cavity of dogs and cats. In association with bites and other types of contact with animals, these bacteria can be transmitted to humans and cause infections[45,50,116]. Upon suspicion of infection, a sample for bacterial culture is taken and sent to the clinical bacteriological laboratory, which have their primary task of providing adequate information about the significant microorganisms that may play a role in the progress of the infection[41]. Proper identification and antibiotic susceptibility testing of the bacteria are of great importance for treatment and diagnostics[128].

Cultivation and biochemical characterization have for a long time been used as the standard diagnostic procedure in bacteriological laboratories. However, in recent years these classical methods have been complemented with modern diagnostic tools, such as nucleic acid–based technologies (DNA sequencing, PCR) and MALDI-TOF MS, which can improve the diagnosis of bacterial infections after animal bites[129].

Between 2005-2010, we collected and isolated 22 *Capnocytophaga* species from blood cultures and wound infections. We used this collection to test the performance of MALDI-TOF MS in the identification of *Capnocytophaga* species and compared its capacity with other established methods in our laboratory. We were aware about the limitations of classical methods and the usefulness of MALDI-TOF in clinical bacteriology had been proven for many other bacterial species.

Classical methods are less efficient when diagnosing *Capnocytophaga* species, due to the slow growth of these bacteria. In paper I we showed that MALDI-TOF was superior to conventional tests in the identification of almost all strains to the species level. A combination of bacterial cultivation and MALDI-TOF seems to be the most efficient way to diagnose *Capnocytophaga* species in clinical laboratories. In particular, MALDI-TOF is a rapid and cost effective method but is strongly dependent on the database and available reference spectra for the actual species to be identified.

A reasonable question to ask at this point is why it is important to correctly identify bacteria to the species or sub-species level? As a bacteriologist, the natural answer is that it provides important information that not only will inform the clinician about various treatment options, but also increase the general knowledge about bacteria and diseases caused by bacteria. In fact, a correct identification may be crucial for the initiation of the correct antibiotic therapy. In addition, a certain species can be linked to a particular disease or condition. This can be illustrated by *Yersinia intermedia*, a bacterium with low pathogenic potential, which is similar to *Yersinia pestis* the causative agent behind the Black Death (Plague)[130]. Finally, in our case it is important to correctly identify *C. canimorsus*, which is associated with sepsis whereas the closely related *C. cynodegmi* is significantly less pathogenic and mainly associated with wound infections.

4.2 Study II

Rationale: This study was carried out for several reasons: a) Resistance to the complement-system is considered to be an important virulence factor in Gram-negative bacteria[82], b) Interactions between *C. canimorsus* and human serum have been studied but there were uncertainties whether *C. canimorsus* is resistant or sensitive to normal human serum; c) There was no data on the interaction between *C. cynodegmi* and human serum and this study could thus fill a knowledge gap in the field.

Aim: To study the growth of *C. canimorsus* and *C. cynodegmi* in human whole blood and serum.

Main results: The results from this study showed that both *C. canimorsus* and *C. cynodegmi* were killed in the presence of human whole blood and serum. Both the reference strains and all tested clinical strains of *C. canimorsus* and *C. cynodegmi* were rapidly killed in NHS. Further experiments showed that *C. canimorsus* and *C. cynodegmi* were killed by different complement pathways. *C. canimorsus* was killed in a classical pathway dependent manner, whereas *C. cynodegmi* was killed by a mechanism that likely involved both the alternative and classical pathways. Both *C. canimorsus* and *C. cynodegmi* were killed in MBL deficient serum, which indicated that the lectin pathway was probably not involved in complement-mediated killing of *Capnocytophaga*. In addition, sera from three patients with a history of sepsis caused by *C. canimorsus* were tested against both the autologous strain (*C. canimorsus* isolated from the patient's blood) and against another *C. canimorsus* exhibited good killing capacity.

Discussion: Resistance to complement is an important virulence factor in gram negative bacteria and to find out if *Capnocytophaga* species was carrying any complement resistance mechanisms, we studied the growth of Capnocytophaga in the presence of whole blood and normal human serum. Unexpectedly, we found that all isolates tested were readily killed by both whole blood and NHS and that the killing was complement-mediated. This was contradicting a previous report where *C. canimorsus* had been described as resistant to human serum and complement[114]. Since the complement system has an important role in the defence against invaders it is important not to define "sensitive-bacteria" as "serum-resistant". In addition, none of the *Capnocytophaga* species strains could survive or grow in MBL deficient serum, which indicate that the lectin pathway has a minor role in inhibiting the growth of *Capnocytophaga* species. Inhibition of the classical pathway favoured growth of *C. canimorsus*, which points to a role for IgM. Interestingly, there is possible relationship between *C. canimorsus* sepsis and serum levels of IgM. In particular this could be relevant for asplenic patients, which constitute 30% of the clinical cases of *C. canimorsus* sepsis. Asplenic patients have in general low numbers of IgM memory B cells[117,131–133], which could make them more susceptible to infections caused by *Capnocytophaga*. We performed some pilot-experiments with sera from patients with congenital asplenia and sera from two out of three individuals failed to kill *C. canimorsus* (unpublished) Since we know that animal *Capnocytophaga* species are mostly sensitive to human serum it may be of importance to check the humoral immune status and concentration of IgM memory B cells of *C. canimorsus* sepsis-patients. It is well known that humoral immunodeficiencies, haematological disorders alcohol consumption and metabolic abnormalities have a negative impact on the humoral immune status[134–136]. It would be interesting and relevant to study the humoral immune status

of *C. canimorsus* patients, since it could explain the pathogenicity of *C. canimorsus* and its progress to fulminant sepsis. Finally, our study suggests that animal *Capnocytophaga* species are sensitive to normal human serum and we failed to find any complement resistance mechanisms. Importantly, health care staffs, people with closes contact with animals and in particular people with an age over 40, asplenic individuals as well as alcoholics should be aware about *C. canimorsus* infections.

Study III

Rationale: In paper I, one strain could not be unequivocally identified to either *C. canimorsus* or *C. cynodegmi*, which lead us to hypothesize that there, could be additional species in the Capnocytophaga family.

Aim: To perform whole genome sequencing to study the phylogenetic relationships between *Capnocytophaga* strains.

Main results: Whole genome sequencing of all strains in our collection, including the recently described *C. canis*, was performed. First, phylogenetic analysis revealed that our previously "unidentified strain" W13 clustered together with the recently discovered *C. canis.* DNA-DNA hybridization (DDH) analysis showed that W13 had high probability to be a *C. canis*. Next, we found that that three strains (W5, W10 and W12) that previously had been identified as *C. cynodegmi* formed a sister clade to the proper *C. cynodegmi*-group in the phylogenetic tree.

DDH-analysis revealed that the strains W5, W10 and W12 had low probability we concluded that, (0.01–0.09%) to belong to any of the already known *Capnocytophaga* species. Therefore, W5, W10 and W12 represents yet another animal bite associated *Capnocytophaga* species, given the putative name "*C. stomatis*" by us.

A detailed phenotypic characterization of the novel species showed that W5, W10 and W12 as well as W13, just like other *Capnocytophaga* species, required CO_2 for growth, and had similar cell morphology in gram staining as other *Capnocytophaga* species. However, the colony morphology differed from the reference strains with regard to size, colour and shape.

Discussion: In study I we showed the strengths and limitations of different methods in identification of *Capnocytophaga* species. The genetic similarities within the *Capnocytophaga species* family makes it difficult for these tools to distinguish closely related species. We faced some difficulty when attempting to identify W13. However, our genomic analysis showed that this strain belongs to the recently described species of the *C. canis.*

Additionally, three other isolates were shown to constitute its own group for which we proposed the name *C. stomatis*. This study is the first describing the isolation of two novel species, *C. canis* and *C. stomatis*, from wounds caused by animal-bites. What is the significance of these discoveries? There are several answers to this question. i) A correct classification of microorganisms is simply is one way to describe our environment; ii) these findings expand our view of the normal flora of pet animals and illustrate the complexity of this microbial community; iii) finally, a correct classification of organisms is important from an epidemiological perspective since it can contribute to link specific microorganisms to certain conditions and diseases. Given the rapid developments in clinical microbiology, we anticipate further studies on the diagnosis and pathogenicity of animal bite associated *Capnocytophaga* species.

Study IV

Rationale: It is of great importance to initiate appropriate antibiotic therapy to treat wound- and systemic infections related to animal bites.

Aim: To determine the antibiotic profiles against animal *Capnocytophaga* species.

Main results: Antimicrobial susceptibility testing by the Etest method confirmed that *C. canimorsus* strains are susceptible to penicillin, cefotaxime, ceftazidime, imipenem and doxycycline. Importantly, two strains of *C. cynodegmi* and two strains of *C. stomatis* had penicillin-resistant phenotypes. The web-based tool, Resfinder, confirmed the presence of a class D beta-lactamase gene in these strains, which corresponds to phenotypic penicillin resistance.

Discussion: In this study, we describe the phenotypic and the genotypic resistance profiles of *Capnocytophaga* species, isolated from wound and blood infections related to dog and cat bites/contact. Normally prophylactic antibiotics for uncomplicated wounds are not recommended, but in the case of high risk to develop infections, first-line oral antibiotic therapy is recommended and even intravenous antibiotics may be given for treatment of severe infections[33,37,42]. The beta-lactam antibiotic amoxicillin-clavulanate is widely used and considered as a first-line antibiotic treatment for dog or cat bite wounds. The risk of development of resistance among many different medically important microorganisms is known. Here we show that *C. cynodegmi* and *C. stomatis* can be resistant to amoxicillin-clavulanate and the potential consequences of this finding may be difficult to answer today, but it is possible that multi-drug resistant *Capnocytophaga* species may occur in the future. Combined, the potentially high virulence connected with *Capnocytophaga* species together

with a risk for antibiotic resistance, we suggest that bacterial cultures always are obtained after cat and dog bites and that broad empirical antibiotic therapy is initiated, especially in high risk patients.

5 CONCLUSIONS

Study I

MALDI-TOF with a proper reference database outperforms biochemically based tools, such as VITEK2, in the identification of animal bite associated *Capnocytophaga* species, in particular for identification to the species level. MALDI-TOF analysis is an excellent complement to classical bacteriological methods within clinical microbiology laboratories by virtue of its low cost, user-friendliness and speed.

Study II

C. canimorsus and *C. cynodegmi* are susceptible to human whole blood and human serum in a complement-dependent manner.

Study III

By using phylogenetic analyses and DNA-DNA hybridization we could identify a novel *Capnocytophaga* species, for which we proposed the name *Capnocytophaga stomatis*. We also found the recently described *C. canis* in a clinical wound specimen, suggesting that this species can be transmitted to humans from both dogs and cats. Combined, these findings emphasize the complexity of animal bite infections.

Study IV

Both *C. cynodegmi* and *C. stomatis* can be resistant to the first line antibiotic treatment amoxicillin-clavulanate. Genomic analysis revealed that these strains carried a class D beta-lactamase gene, with carbapenemase activity, which most likely explained the phenotypic resistance patterns with reduced susceptibility to amoxicillin-clavulanate, cefalosporins and carbapenems.

6 FUTURE PLANS AND PERSPECTIVE

I will here outline a number of feasible and obvious follow-up studies:

- (1) The database for MALDI-TOF spectra should be improved for all *Capnocytophaga species* by the addition of a larger collection. This would enable a rapid way to obtain a significantly higher precision in the clinical diagnostic procedures for animal bite associated *Capnocytophaga* infections. In particular, it would be of value to include the recently discovered species *C. canis* and *C. stomatis*.
- (2) I would like to develop selective agar plates for the isolation of *Capnocytophaga species* from animal bite associated wound specimens. This would greatly increase the probability of finding these fastidious bacteria in wound-cultures. Combined, project 1 and 2 outlined here would expand the knowledge on the epidemiology, antibiotic susceptibility and treatment outcomes of various *Capnocytophaga*-related infections.
- (3) Since we still think there is a significant under-diagnosis of *Capnocytophaga canimorsus* in sepsis, I would like to investigate if it is possible to improve the growth of *Capnocytophaga* species in blood culture systems that are commercially available. The rationale is based on the possibility that not all complement activity is blocked in blood culture flasks. If there is residual complement activity left, it is possible that further optimization of *Capnocytophaga* growth is possible.
- (4) I would also like to study what makes *C. canimorsus* much more virulent than other *Capnocytophaga* species. I would like to study the immune response of normal blood to these bacteria. Does the immune response to *C. canimorsus* differ from the immune response to other *Capnocytophaga* species i.e. *C. cynodegmi, C. canis* and *C. stomatis*?
- (5) Finally, I find it fascinating that children appears to be resistant to *C. canimorsus* sepsis, despite the many bites and scratches that occur in this age-group. It is tempting to hypothesize that there is an explanation to find in the immune system of children. It would be interesting to monitor the presence and level of antibodies against *C. canimorsus* in children vs adults. In fact, we started to work on the serology of *Capnocytophaga* species by using serum from patients with a history of *Capnocytophaga* sepsis. This could be one way forward to identify unique antigens for exposure to *C. canimorsus* and *C. cyndodegmi,* respectively. Serology could also provide an interesting tool to study the true epidemiology of infections with these bacteria.

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